

Characterisation of the Adiponectin Receptors: Differential cell-surface expression and temporal signalling profiles of AdipoR1 and AdipoR2 are regulated by the non-conserved N-terminal trunks.

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Abstract

The adiponectin axis regulates cardiometabolic and inflammatory tone making it an attractive therapeutic focus. Rudimentary understanding of the adiponectin receptors, AdipoR1 and AdipoR2, constrains our ability to target these atypical seven trans-membrane proteins. Here, we aimed to further elaborate the molecular details governing cell-surface expression and signal transduction by transient expression of AdipoR1 or AdipoR2 in HEK293 cells. Following serum starvation, adiponectin reduced cell-surface expression of both receptors, consistent with internalisation, and promoted phosphorylation of downstream effectors. Temporal phosphorylation profiles differed with AdipoR1 and AdipoR2 transduced signals peaking at 15 min and 24 h. Analysis of receptor chimeras showed that the non-conserved N-terminal trunks (AdipoR1₍₁₋₇₀₎ and AdipoR2₍₁₋₈₁₎) define the temporal signalling profiles and contain multiple regions that promote or inhibit cell-surface expression, respectively. These findings highlight the importance of the non-conserved N-terminal trunks and demonstrate that cell-surface expression of AdipoR1 and AdipoR2 is required for effective coupling to downstream effectors.

Keywords: Signal Transduction, Receptor, Adiponectin

1. Introduction

Adiponectin is a key adipokine with demonstrated anti-diabetic, anti-inflammatory and anti-atherogenic properties (Scherer, 2006). Adiponectin's pleiotropic effects are mediated through two atypical 7 transmembrane (7TM) domain receptors termed AdipoR1 and AdipoR2 (Hickman and Whitehead, 2012; Yamauchi et al., 2003). Empirical evidence demonstrates that AdipoR1 and AdipoR2 have intracellular N-termini and extracellular C-termini (Yamauchi et al., 2003) unlike most other 7TM proteins including the G-protein coupled receptors (GPCRs). *In vivo* and *in vitro* studies have demonstrated that AdipoR1 and AdipoR2 mediate the effects of adiponectin via activation of a number of signalling molecules such as AMPK, PPAR α , ERK and P38MAPK (Cong et al., 2007; Deepa and Dong, 2009; Shibata et al., 2005; Wijesekara et al., 2010; Yamauchi et al., 2007). Coupling to these downstream effectors has been shown to be modulated by proteins that interact with the cytoplasmic, N-terminal domains of both AdipoR1 and AdipoR2, such as APPL1, RACK and protein kinase CK2 (Deepa and Dong, 2009; Heiker et al., 2009; Xu et al., 2009), or with the non-conserved N-terminal trunk of AdipoR1, namely ERp46 (Charlton et al., 2010).

Emerging evidence indicates that obesity-related diseases are characterised not only by hypoadiponectinemia but also by adiponectin resistance at the level of the adiponectin receptors (Bruce et al., 2005; Chen et al., 2005; Kintscher, 2007; Lara-Castro et al., 2008; Mullen et al., 2007; Rodríguez et al., 2008). Thus, a greater understanding of the molecular processes required to facilitate efficient adiponectin receptor coupling to intracellular signalling pathways may be expected to provide new insights into pathophysiological events and the identification of novel therapeutic approaches.

We recently reported that under steady-state conditions (no serum starvation) the cell-surface expression of AdipoR1 and AdipoR2 differs. AdipoR1 is enriched in the plasma membrane

whilst AdipoR2 is more abundant in the ER (Keshvari et al., 2013). We also demonstrated that this difference is due to the non-conserved N-terminal trunks of AdipoR1 and AdipoR2 (Keshvari et al., 2013). In the current report we have extended these studies by performing further characterisation of the molecular features governing the cell-surface expression and subsequent coupling to downstream signalling effectors of AdipoR1 and AdipoR2. Our results demonstrate that the non-conserved N-terminal trunks dictate the cell-surface expression and temporal signalling profiles of AdipoR1 and AdipoR2.

2. Materials and methods

2.1. Reagents and antibodies

Reagents were from Sigma–Aldrich (Castle Hill, Australia) unless otherwise stated. Tissue culture reagents were from Invitrogen (Mount Waverley, Australia). Primary antibodies against HA and Sodium Potassium ATPase were from Covance (Washington, USA) and Abcam (Melbourne, Australia) respectively. Primary antibodies against AdipoR1 and AdipoR2 were as described (Charlton et al., 2010). Secondary antibodies were from Life Technology (Invitrogen). Molecular biology reagents were from New England Biolabs (Ipswich, MA, USA) or Promega Corporation (Madison, WI, USA). Serum from adult WT and adiponectin knockout mice (Maeda et al., 2002) was collected in accordance with ethics approval from the animal ethics committee of the University of Queensland.

2.2. Molecular biology

Original constructs encoding C-terminally epitope-tagged (HA) human AdipoR1 and AdipoR2 were as described (Charlton et al., 2010). Chimeric and truncated receptor constructs were generated as described (Keshvari et al., 2013). AdipoR1 and AdipoR2 mutants were generated by QuikChange site-directed mutagenesis (Agilent Technology, CA, USA). Mutations were confirmed by DNA sequencing (Sanger method). Chinese Hamster Ovary (CHO) cells or Human Embryonic Kidney 293 (HEK293) cells were transfected using Lipofectamine PLUS (Invitrogen) according to the manufacturer's instructions. Transfection efficiency was typically around 70% and cells were analysed 24-48 h post-transfection.

2.3. Flow cytometry analysis

Flow cytometry was performed to determine the percentage of transfected cells that were expressing AdipoR1 or AdipoR2 at the cell-surface. Flow cytometry was carried out using a CyAn™ ADP Analyser (Beckman Coulter, Sydney, Australia) and FlowJo software. Briefly, HEK293 cells were washed with cold PBS and stained with HA antibody. For analysis of permeabilised cells, cells were incubated in 0.1% Saponin for 15 min prior to blocking. After washing with PBS, cells were stained with an AlexaFluor 488-conjugated secondary antibody (Invitrogen). Cells were then lifted non-enzymatically and 80,000 events were analysed by flow cytometer. For all experiments, mean fluorescence intensity (MFI) values were calculated by subtracting secondary only staining from specific anti-HA staining.

2.4. Plate-based determination of cell-surface expression of AdipoR1 and AdipoR2

Quantitative measurement of total and cell-surface expression of AdipoR1 and AdipoR2 was performed, in permeabilised and non-permeabilised cells respectively, using a plate-based assay as described (Keshvari et al., 2013). Briefly, parental HEK293 cells or transfected HEK293 cells were incubated in either the presence or absence of serum at 37°C overnight. Cells were incubated in 100% ice-cold methanol for 5 min to permeabilise (for measurement of total receptor levels) or left non-permeabilised (for determination of receptors at the cell-surface). Cells were then stained with HA antibody followed by fixation with 4% Paraformaldehyde. Cells were stained with an AlexaFluor 488-conjugated secondary antibody. Signals were detected using the POLARstar Omega plate-reader (BMG Labtech, Offenburg, Germany).

2.5. Immunofluorescence microscopy

Immunofluorescence microscopy of permeabilised and non-permeabilised cells was performed as described (Keshvari et al., 2013) affording details of the subcellular distribution of the receptors and the cell-surface expression respectively. Images were taken using a Delta Vision OMX microscope (Applied Precision, GE Healthcare, Washington, USA).

2.6. Akt, ERK and p38MAPK phosphorylation assays

Parental HEK293 cells or transfected HEK293 cells were serum-starved overnight then stimulated with recombinant human globular adiponectin (Prospec Protein Specialists, USA) or vehicle for 15 min, 1 h or 24 h. Phosphorylation of Akt, ERK and p38MAPK was measured using AlphaScreen SureFire kits essentially as described (PerkinElmer Life and

Analytical Sciences, Waltham, MA, USA). Plates were read using a POLARstar Omega plate reader. Background signals were determined by treatment of cells with the Akt1/2 kinase inhibitor, U0126 (ERK inhibitor) or SB203580 (p38MAPK inhibitor) and were subtracted to give specific phospho-signals for Akt, ERK and p38MAPK respectively.

2.7. SDS-PAGE/Western blotting of AdipoR1 and AdipoR2

Western blotting of particulate fractions (enriched for the ER and PM that contain greater than 90% of total cellular AdipoR1 and AdipoR2) was performed on parental and transfected cells as described (Charlton et al., 2010).

2.8. Statistical analysis

Data are presented as mean \pm SEM. Significance was determined using one way ANOVA followed by Tukeys test with statistical significance defined as $p < 0.05$.

3. Results

3.1. Serum starvation increases the cell-surface expression of AdipoR1 and AdipoR2.

We previously reported that under steady-state conditions the subcellular localisation of AdipoR1 and AdipoR2 differed with around 50% of AdipoR1 present on the cell-surface whilst AdipoR2 was localised predominantly at the ER (Keshvari et al., 2013). We subsequently demonstrated that the non-conserved, N-terminal trunks of AdipoR1₍₁₋₇₀₎ and AdipoR2₍₁₋₈₁₎ underpinned these differences (Keshvari et al., 2013). To extend these studies we first examined the cell-surface expression of transiently expressed, C-terminally HA-tagged AdipoR1 and AdipoR2 (Keshvari et al., 2013) in serum-starved or non-starved HEK cells using flow cytometry and plate-based assays as well as high resolution microscopy. Flow cytometry was used to determine the number of cells with detectable cell-surface expression of AdipoR1 or AdipoR2. Serum starvation did not affect the number of cells with AdipoR1 on the cell-surface however the number of cells with detectable cell-surface expression of AdipoR2 was significantly increased following serum starvation (Fig1A). A complementary plate-based assay was used to determine total and cell-surface levels of

AdipoR1 and AdipoR2. This approach revealed significantly increased cell-surface expression of both AdipoR1 and AdipoR2 following serum starvation (Fig 1B). Finally, qualitative analysis by high resolution confocal microscopy suggested that the cell-surface expression of AdipoR1 was increased in cells following serum starvation. We were unable to detect cell-surface expression of AdipoR2 in non-permeabilised cells in steady-state or serum-starved cells which probably reflects a limitation of this approach (Fig 1G-J). These results extend our previous findings (Keshvari et al., 2013) by showing that serum starvation results in an increase in the proportion of AdipoR1 or AdipoR2 that is expressed on the cell-surface with the latter resulting in an increase in the number of cells with detectable cell-surface levels of AdipoR2. Notwithstanding, the levels of AdipoR2 on the cell-surface of serum starved cells are still relatively limited compared to those of AdipoR1.

3.2. Adiponectin reduces cell-surface expression of AdipoR1 and AdipoR2.

We next went on to investigate the effect of serum, and more specifically adiponectin, on receptor cell-surface expression. Following overnight serum starvation cells overexpressing either AdipoR1 or AdipoR2 were incubated with 10% fetal bovine serum (FC) for 30, 60, 90, 120 and 240 min. Analysis by flow cytometry (Fig 2A-B) and microscopy (Fig 2C-J) indicated that cell-surface expression of both AdipoR1 and AdipoR2 was reduced by 60% and 90% after 30 min. To investigate the role of adiponectin more specifically we then used serum from wild-type (WT) or adiponectin knockout ($Adn^{-/-}$) mice. Serum from WT mice promoted similar effects as the FC, whilst serum from $Adn^{-/-}$ mice was without effect (Fig 2A-B). Similar results were obtained following treatment with 2.5 μ g/ml globular adiponectin (gAd) (Fig 2A-B). These results are consistent with the adiponectin-mediated internalisation of AdipoR1 and AdipoR2 reported previously (Almabouada et al., 2013), and suggest this is a specific, ligand-mediated event.

3.3. *Overexpression of AdipoR1 and AdipoR2 enhances adiponectin stimulated Akt, ERK and P38MAPK phosphorylation.*

We next examined the effects of AdipoR1 and AdipoR2 overexpression on adiponectin-stimulated phosphorylation of key signalling molecules implicated in mediating adiponectin's effects, namely Akt (Cui et al., 2011; Wijesekara et al., 2010), ERK (Lee et al., 2008; Wijesekara et al., 2010) and P38MAPK (Charlton et al., 2010; Tang et al., 2007). In the parental HEK293 cells endogenous levels of AdipoR1 and AdipoR2 were undetectable by standard Western blot whereas transfected AdipoR1 and AdipoR2 were readily detected (Fig 3A & B). Parental cells or cells overexpressing AdipoR1 or AdipoR2 were treated with recombinant gAd for 15 min or 24 h (based on preliminary timecourse experiments - data not shown) to determine acute and long-term effects. Under these experimental conditions there was no detectable adiponectin-stimulated phosphorylation of Akt, ERK, or p38MAPK in the parental cells. However, significant and maximal phosphorylation of Akt, ERK, and p38MAPK occurred after 15 min in AdipoR1 expressing cells, and after 24 h in AdipoR2 overexpressing cells (Fig 3C-E). We then went on to perform dose response studies, treating cells with increasing concentrations of gAd (0.5 – 5.0 µg/ml) for either 15 min or 24 h. After 15 min, the phospho-Akt dose response was similar in AdipoR1 and AdipoR2 overexpressing cells (Fig 3F), although AdipoR1 typically mediated 10-20% higher phosphorylation than AdipoR2. However, after 24 h the phospho-Akt dose response was markedly different. AdipoR2 promoted robust Akt phosphorylation with as little as 0.5 µg/ml gAd whilst AdipoR1 was without effect at concentrations up to 1.5 µg/ml gAd (Fig 3I). Coupling to ERK and p38 MAPK also showed different characteristics with AdipoR1 exhibiting greater transduction than AdipoR2 at 15 min, especially at gAd concentrations of 2.0 µg/ml or higher (Fig 3G & H). These differences became less marked after 24 h (Fig 3J & K) with phosphorylation of P38MAPK being constitutively higher in AdipoR2 cells compared with

AdipoR1 cells (Fig 3K). Collectively these results provide evidence that there are fundamental differences between signalling emanating from AdipoR1 and AdipoR2, most notably the difference in temporal profiles with AdipoR1 acting more acutely than AdipoR2.

3.4. The subcellular localisation of AdipoR1 and AdipoR2 is governed by multiple domains.

We previously reported that the non-conserved N-terminal trunks of AdipoR1₍₁₋₇₀₎ and AdipoR2₍₁₋₈₁₎ underpinned the observed differences in cell-surface expression (Keshvari et al., 2013). To investigate this further we generated and characterised the cell-surface expression of a number of chimera (Fig 4A). Analysis by flow cytometry demonstrated a striking profile, with increasing inclusion of the non-conserved trunk of AdipoR2 reducing cell-surface expression whilst the converse was observed upon increasing content of the non-conserved trunk of AdipoR1 (Fig 4B). These findings suggest there is not a single region or motif within the non-conserved trunks that underpins the different cell-surface expression profiles of AdipoR1 and AdipoR2 but that multiple regions contribute to these differences.

Next we characterised the signalling properties of the two chimera in which the entire non-conserved N-terminal trunks had been swapped (termed R1₍₇₀₎R2 and R2₍₈₁₎R1, respectively) (Fig 4A). In cells overexpressing R2₍₈₁₎R1 chimera the temporal profiles of Akt, ERK and p38MAPK phosphorylation showed peak phosphorylation at 24 h whereas in cells overexpressing R1₍₇₀₎R2 chimera phosphorylation peaked at 15 min (Fig 4C-E). These profiles closely resembled those of AdipoR2 and AdipoR1 respectively (see Fig 3A-C), indicating that key differences in the temporal signalling profiles of AdipoR1 and AdipoR2 are dictated by the non-conserved N-terminal trunks.

3.5. Cell-surface expression and downstream signalling of AdipoR1 and AdipoR2 is regulated by conserved F(x)₃F(x)₃F and D(x)₃LL motifs.

Two different motifs within the intracellular N-terminal trunk of AdipoR1 have been shown to be essential for its efficient cell-surface expression (Juhl et al., 2012). Mutation of an acidic di-leucine motif ($_{106}\text{D}(\text{x})_3\text{LL}$) or a putative ER exit motif ($_{121}\text{F}(\text{x})_3\text{F}(\text{x})_3\text{F}$), which are known to regulate trafficking of GPCRs (Bermak et al., 2001; Schulein et al., 1998), resulted in inhibition of cell-surface expression of AdipoR1 (Juhl et al., 2012). Alignment of the primary amino acid sequence of AdipoR1 and AdipoR2 from multiple species revealed conservation of these motifs between AdipoR1 and AdipoR2 (Fig 5A) leading us to speculate that these motifs would also be required for efficient cell-surface expression of AdipoR2. To test this we generated and characterised the expression of AdipoR2, and AdipoR1, mutants in which the key residues were mutated to alanine (termed R1/R2-FFF or R1/2-DLL where each of the residues was mutated to A). Flow cytometry of serum starved cells revealed a significant reduction in the cell-surface expression of AdipoR1-FFF and AdipoR1-DLL as well as AdipoR2-FFF and AdipoR2-DLL constructs compared to the WT receptors (Fig 5B & F). Furthermore, these constructs exhibited reduced adiponectin-stimulated phosphorylation of Akt (Fig 5C&G), ERK (Fig 5D&H) and P38MAPK (Fig 5E&I). These results indicate that efficient cell-surface expression of AdipoR1 and AdipoR2 is required for adiponectin signal transduction.

4. Discussion

In the current report we have elaborated the molecular details governing differential cell-surface expression and downstream coupling of the adiponectin receptors, AdipoR1 and AdipoR2. We show that serum starvation increases cell-surface expression of both AdipoR1 and AdipoR2 and that, in contrast to serum from WT mice, serum from mice lacking adiponectin fails to reduce receptor cell-surface expression consistent with receptor internalisation. We also demonstrate that the temporal signal transduction profiles differ for AdipoR1 and AdipoR2. Furthermore, we show that these differences are intrinsically

coupled to their cell-surface expression profiles and their non-conserved, N-terminal trunks which appear to contain multiple regions that promote or reduce cell-surface expression respectively.

Previously, we reported that AdipoR1, but not AdipoR2, interacted with ERp46 via its non-conserved N-terminal trunk (Charlton et al., 2010). Further investigations revealed that the non-conserved N-terminal trunks of AdipoR1 and AdipoR2 also dictated the cell-surface expression of AdipoR1 and AdipoR2, with robust cell-surface expression of AdipoR1 but not AdipoR2 observed under steady-state conditions in CHO cells (Keshvari et al., 2013). To address the impact of this difference on coupling to downstream signalling events we first characterised the effects of serum starvation followed by serum replacement on cell-surface expression of the receptors. Serum withdrawal resulted in increased expression of both AdipoR1 and AdipoR2 although AdipoR2 still exhibited significantly lower cell-surface expression than AdipoR1. The addition of serum or gAd reduced cell-surface expression of both receptors and microscopy suggested that this decrease reflected classic ligand-dependent internalisation consistent with a previous report (Almabouada et al., 2013). Our findings using serum from wild-type and adiponectin KO mice suggest that, at least under these experimental conditions, no other circulating factors, such as members of the CTRP family (Schaffler and Buechler, 2012), are able to promote internalisation of the adiponectin receptors.

Since the seminal discovery of the adiponectin receptors by Kadowaki and colleagues (Yamauchi et al., 2003) a large body of evidence has accumulated which indicates that adiponectin-stimulated activation of intracellular signalling pathways via the adiponectin receptors is highly variable across cell-types and tissues (Hickman and Whitehead, 2012). The precise mechanisms for this variability remain relatively poorly understood but a number

of factors are likely to contribute. For example, the expression levels of AdipoR1 and AdipoR2 differ across tissues and cell-types (Yamauchi et al., 2003) as do the expression levels of proteins that have been shown to interact with the receptors and modulate downstream signalling (Heiker et al., 2010). Moreover, the adiponectin receptors exhibit different binding properties (Yamauchi et al., 2003) and investigators have used a range of different recombinant multimeric or globular forms of adiponectin (Hickman and Whitehead, 2012). Whilst all of these factors are likely to contribute to differential activation of intracellular signalling pathways it is also noteworthy that relatively few studies have characterised signalling emanating specifically from AdipoR1 and AdipoR2 under tightly controlled conditions making this an area where our understanding remains particularly rudimentary. Moreover, inspection of the literature reveals several major caveats. For example, in the original report from Yamauchi and colleagues AdipoR2 was identified by sequence homology to AdipoR1 and this probably explains why a truncated form lacking the non-conserved N-terminal trunk was cloned and characterised (Yamauchi et al., 2003). We previously reported that a similar truncated construct exhibits increased cell-surface expression, compared with full-length AdipoR2 (Keshvari et al., 2013), and, in light of the current findings, we predict that it would also show altered signalling profiles although this remains to be determined.

In the present report we used gAd to investigate signalling mediated via AdipoR1 or AdipoR2 constructs in HEK293 cells. This recombinant form of adiponectin has been used widely by others, as it represents a more homogenous, less variable product than full-length multimeric adiponectin (Hickman and Whitehead, 2012) whilst HEK293 cells have been shown to be a suitable cell model for investigations into adiponectin signalling (Lee et al., 2008). Interestingly, and in contrast to the findings from Lee and colleagues (Lee et al., 2008), we were unable to detect endogenous adiponectin receptors in our parental (non-transfected) HEK293 cells and,

consistent with this, we did not observe any response upon treatment with gAd in the parental cells. As expected however, transfection of AdipoR1 or AdipoR2 conferred sensitivity to gAd. We found that acute (15 min) coupling to Akt was similar between AdipoR1 and AdipoR2 under conditions where cell-surface expression of AdipoR1 was typically three-fold higher than for AdipoR2. Moreover, sensitivity of AdipoR2-mediated Akt phosphorylation was significantly greater than that for AdipoR1 after chronic (24 h) treatment, indicating far-greater longevity of the AdipoR2 signal. These results were, at least to some extent, recapitulated when coupling to ERK and p38MAPK was analysed. For both, AdipoR1-mediated phosphorylation peaked at 15 min whilst AdipoR2-mediated phosphorylation peaked at 24 h. To our knowledge, this is the first time such differences in the temporal profiles of adiponectin signalling emanating from AdipoR1 and AdipoR2 have been described.

Detailed analysis of a series of chimeric receptors revealed that the differential cell-surface expression of AdipoR1 and AdipoR2 was defined by multiple regions within the non-conserved N-terminal trunks indicating that no single motif underpinned the observed differences. Moreover, functional investigations revealed that the temporal signalling profiles were also determined by these non-conserved N-terminal trunks.

Two motifs (D(x)₃LL and F(x)₃F(x)₃F) were previously reported to play a role in anterograde trafficking of AdipoR1 (Juhl et al., 2012). We demonstrated that these motifs are conserved within the juxtamembrane region of AdipoR2 and are required for the efficient cell-surface expression of both receptors. We found that mutation of either of these motifs inhibited cell-surface expression and adiponectin-stimulated phosphorylation of Akt, ERK and p38MAPK. These data further highlight the importance of efficient cell-surface expression of AdipoR1 and AdipoR2 for efficient downstream signalling and the complexity of adiponectin receptor trafficking. Further work is required to elaborate the molecular details governing the contribution of the non-conserved N-terminal trunks and the conserved sequence motifs

(including D(x)₃LL and F(x)₃F(x)₃F) and how these, in turn, contribute to the temporal signalling profiles of AdipoR1 and AdipoR2 respectively.

Adiponectin and its receptors are recognised as attractive potential targets for the treatment of cardiometabolic disease (Hickman and Whitehead, 2012). In the current report we have increased our understanding of processes governing cell-surface expression of the adiponectin receptors and demonstrate that efficient cell-surface expression is required to afford sensitivity to adiponectin. We have established that the non-conserved, N-terminal trunks of AdipoR1 and AdipoR2 serve as key determinants of the cell-surface expression and signalling profiles of the receptors. This work provides a foundation for future studies that may aim to enhance adiponectin sensitivity by increasing cell-surface expression of the receptors, particularly AdipoR2.

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6. Figure legends

Fig. 1. Effects of serum starvation on cell surface expression of AdipoR1 and AdipoR2.

(A) Flow cytometry and (B) plate-based analysis of HEK293 cells transiently expressing AdipoR1-HA and AdipoR2-HA constructs. The cell-surface expression (CSE) ratio shows the (A) percentage of transfected cells expressing AdipoR1 or AdipoR2 at the cell-surface and (B) the ratio of cell-surface to total receptor levels. Results are from four independent experiments (* $p < 0.05$). (C-J) immunofluorescent microscopy of transiently expressed AdipoR1-HA or AdipoR2-HA constructs (green) in permeabilised or non-permeabilised CHO cells with or without 16 h serum starvation. Plasma membrane is counterstained with sodium potassium ATPase antibody (red) and nuclei with DAPI (blue).

Fig. 2. Adiponectin reduces the cell-surface expression of AdipoR1 and AdipoR2.

Flow cytometry analysis of cell-surface expression ratio of HEK293 cells transiently transfected with (A) AdipoR1-HA or (B) AdipoR2-HA. Cells were incubated with fetal bovine serum (FC) for 0, 30, 60, 120, 240 min or with serum from wild-type mice (WT) or serum from adiponectin knockout mice (KO) or with gAd (2.5 $\mu\text{g/ml}$) for 30 min. The % MAX represents receptor cell-surface expression in cells without any addition and was set to 100% for AdipoR1 and AdipoR2 respectively. Results are from four independent experiments (* $p < 0.05$). (C-J) immunofluorescent microscopy of AdipoR1-HA and AdipoR2-HA constructs transiently expressed in CHO cells following 0, 30, 60 or 120 min treatment with serum. Plasma membrane is counterstained with sodium potassium ATPase antibody (red) and nuclei with DAPI (blue).

Fig. 3. AdipoR1 and AdipoR2 exhibit different temporal activation profiles. Western blot showing levels of (A) AdipoR1 and (B) AdipoR2 in transfected and parental (non-transfected) HEK293 cells.

Alphascreen analysis of (C) Akt, (D) ERK and (E) P38MAPK phosphorylation in HEK cells transiently expressing AdipoR1 or AdipoR2 constructs incubated with 2.5 $\mu\text{g/ml}$ globular adiponectin (gAd) for 0, 15 min or 24 h. Alphascreen analysis of Akt, ERK and P38MAPK phosphorylation in HEK cells transiently expressing AdipoR1 or AdipoR2 constructs incubated with increasing concentrations of gAd (0 to 5 $\mu\text{g/ml}$) for 15 min (F-H) or 24 h (I-K). Results are from at least four independent experiments. In graphs C-E: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, comparing AdipoR1 or AdipoR2 at different timepoints; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$, comparing AdipoR1 vs

AdipoR2 at the same timepoint; [@]p<0.05, comparing AdipoR1 vs parental cells at the same timepoint. In graphs F-I: *p<0.05, **p<0.01, ***p<0.001 comparing AdipoR1 vs AdipoR2.

Fig. 4. The non-conserved, N-terminal domain of AdipoR1 and AdipoR2 regulates cell-surface expression and signal transduction. (A) schematic representation of generated chimeric constructs. (B) Flow cytometry analysis of cell-surface expression ratio in HEK cells transiently expressing WT AdipoR1-HA, AdipoR2-HA and chimeric constructs, (AdipoR2₍₁₋₄₃₎/R1₍₃₂₋₃₇₅₎-HA, AdipoR2₍₁₋₆₉₎/R1₍₅₈₋₃₇₅₎-HA, AdipoR2₍₁₋₈₁₎/R1₍₇₁₋₃₇₅₎-HA, AdipoR1₍₁₋₃₁₎/R2₍₄₄₋₃₈₆₎-HA, AdipoR1₍₁₋₅₇₎/R2₍₇₀₋₃₈₆₎-HA and AdipoR1₍₁₋₇₀₎/R2₍₈₂₋₃₈₆₎-HA). Alphascreen analysis of (C) Akt, (D) ERK and (E) p38MAPK phosphorylation in HEK cells transiently expressing chimeric AdipoR2₍₁₋₈₁₎/R1₍₇₁₋₃₇₅₎-HA (R2/R1) or AdipoR1₍₁₋₇₀₎/R2₍₈₂₋₃₈₆₎-HA (R1/R2) constructs incubated with 2.5 µg/ml globular adiponectin (gAd) for 0, 15 min or 24 h. Results are from at least four independent experiments (*p<0.05). [#]comparing different genes, same timepoint. ^{\$}comparing selected gene and parental cells, same timepoint.

Fig. 5. F(x)₃F(x)₃F and D(x)₃LL motifs are required for efficient cell surface expression and signal transduction via AdipoR1 and AdipoR2. (A) Sequence alignment of the cytoplasmic N-terminal trunk of AdipoR1 and AdipoR2. Conserved F(x)₃F(x)₃F and D(x)₃LL motifs are highlighted (in grey). (B) Flow cytometry analysis of cell-surface expression ratio in HEK293 cells transiently expressing WT AdipoR1, the F 121, 125, 129A (R1-FFF) and D106, L110, L111A (R1-DLL) mutants. Alphascreen analysis of (C) Akt, (D) ERK and (E) p38MAPK phosphorylation in HEK cells transiently expressing WT AdipoR1, R1-FFF or R1-DLL constructs incubated with 2.5 µg/ml gAd for 15 min. (F) Flow cytometry analysis of cell-surface expression ratio in HEK293 cells transiently expressing WT AdipoR2, the F 132, 136, 140A (R2-FFF) and D117, L121, L122A (R2-DLL) mutants. Alphascreen analysis of (G) Akt, (H) ERK and (I) P38MAPK phosphorylation in HEK293 cells transiently expressing WT AdipoR2, R2-FFF or R2-DLL constructs incubated with 2.5 µg/ml gAd for 24 h. Results are from at least four independent experiments (*** p<0.001).

Fig 1

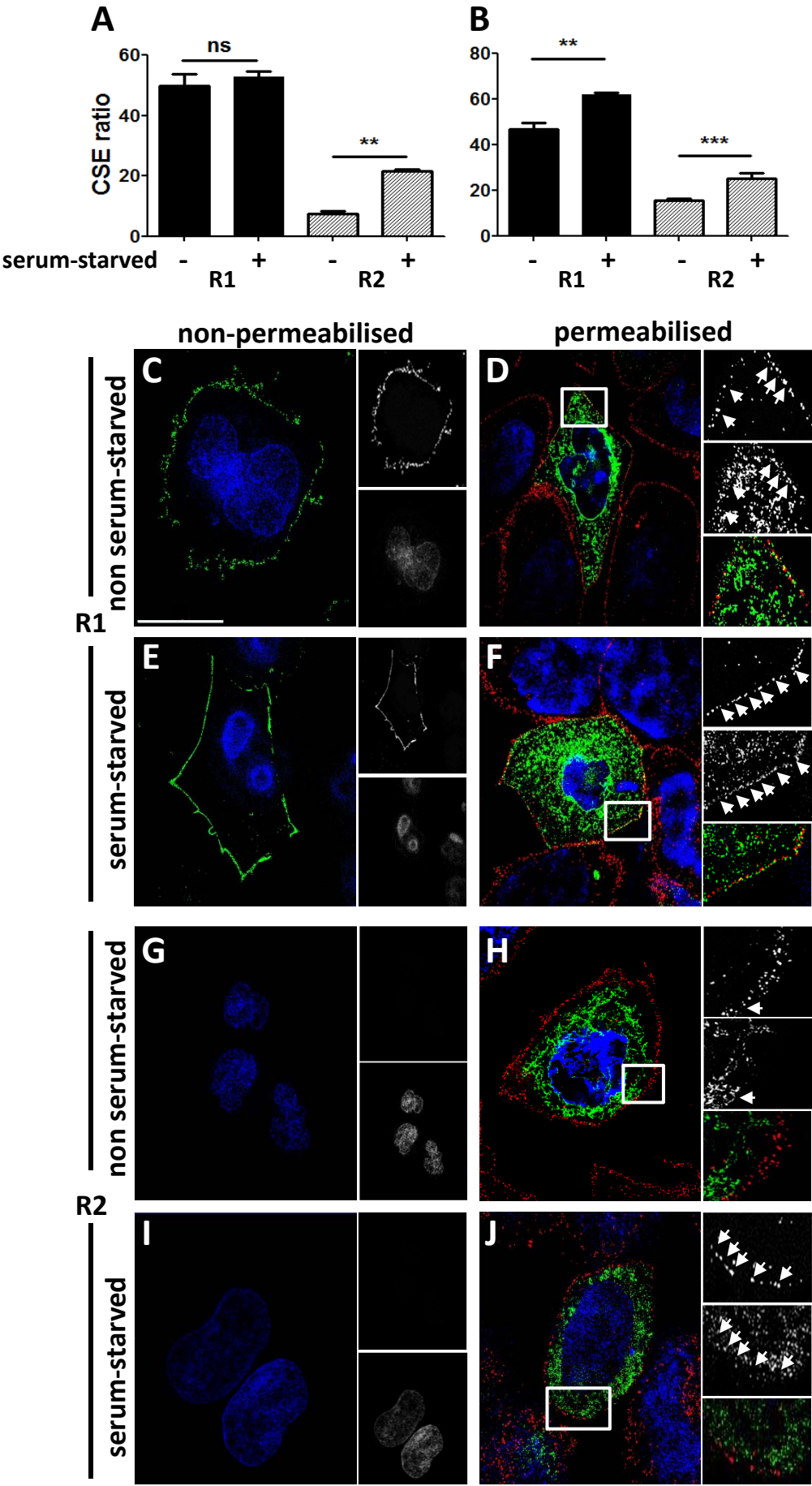


Fig 2

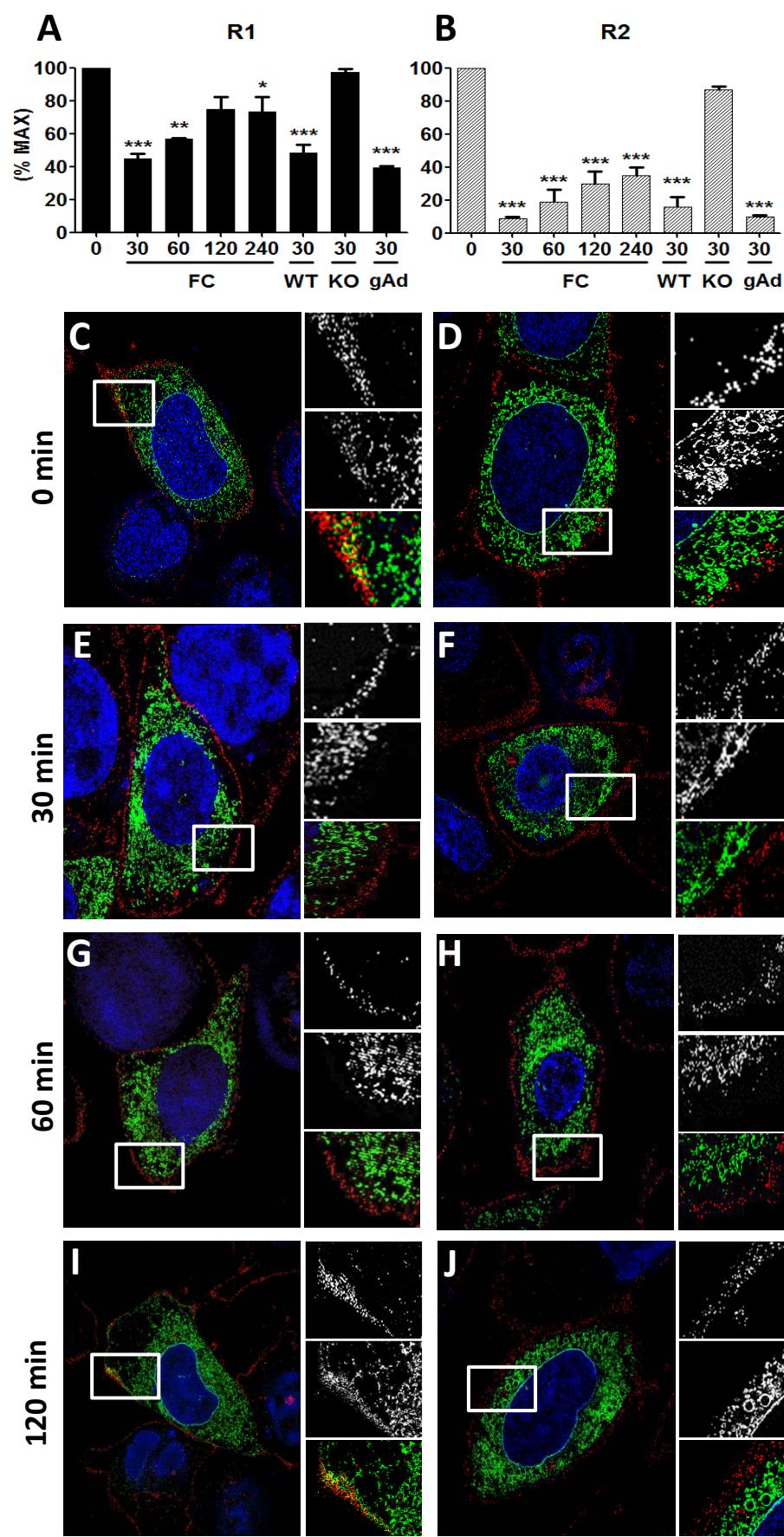


Fig 3

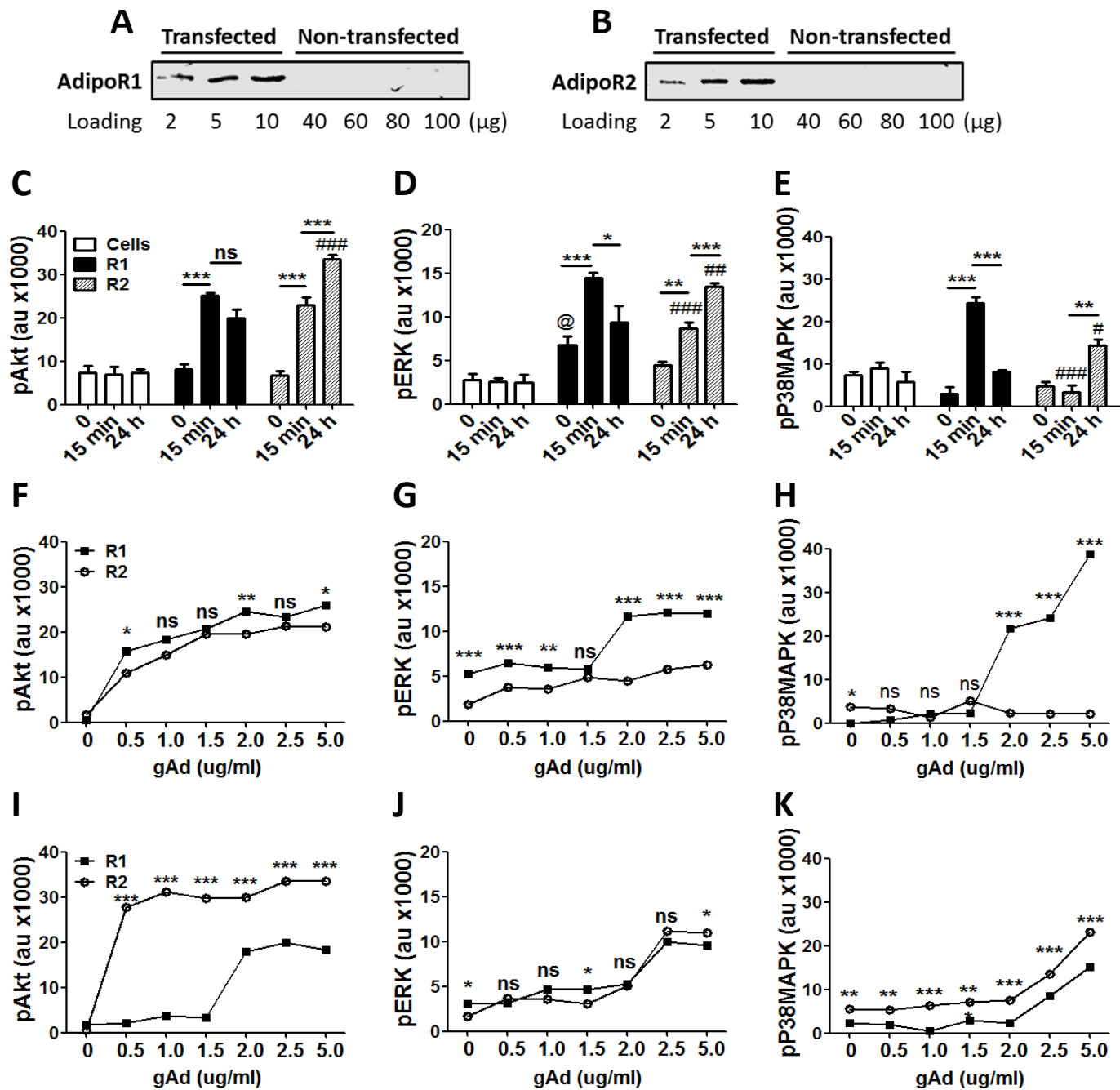


Fig 4

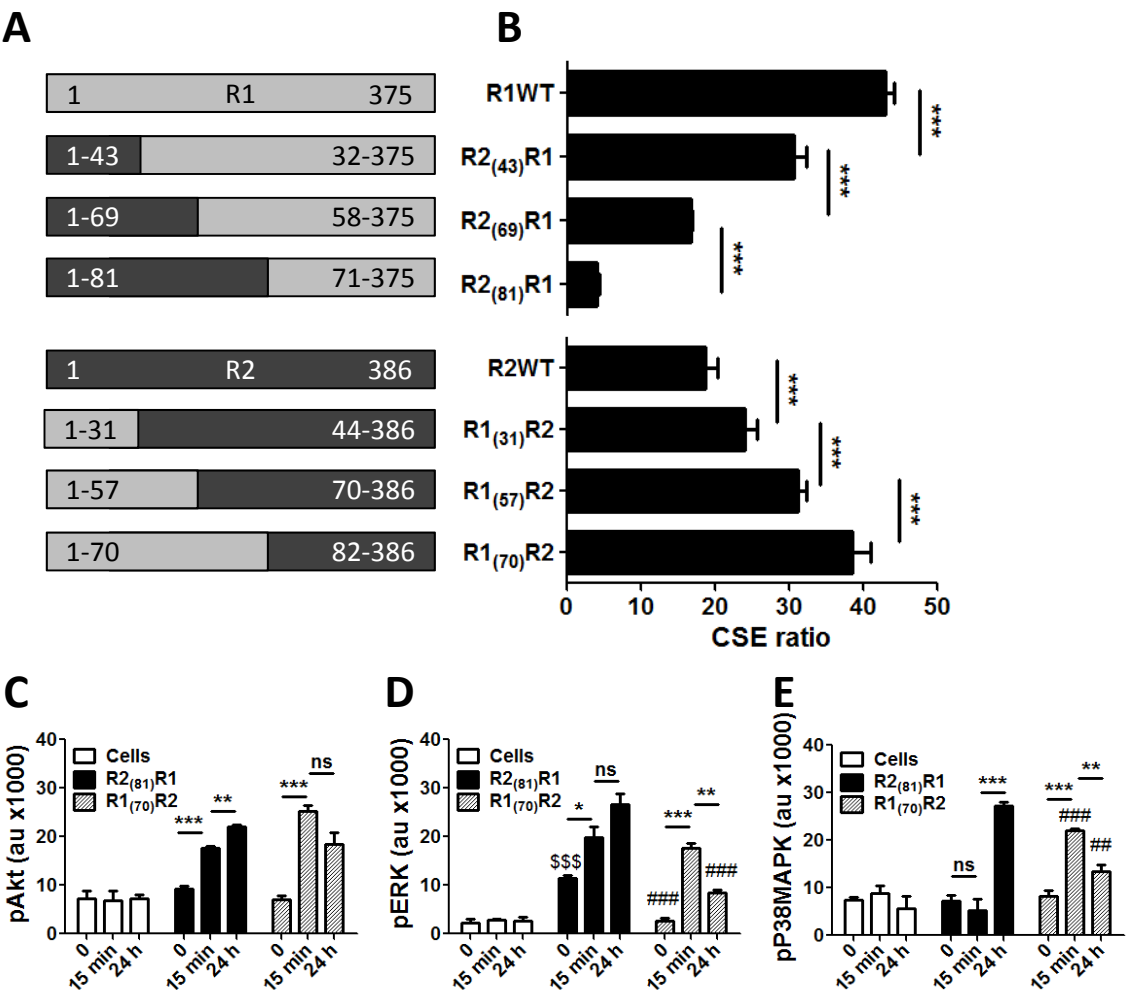
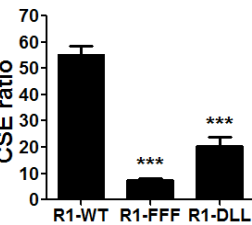


Fig 5

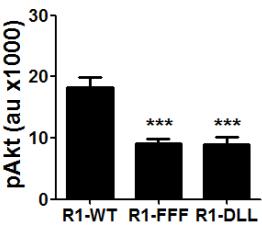
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Mouse R1  -----MSSHKGS----AGAQGNGAPSGNREADTVELAE LGPLLEEK GKRAASSPAKAEEQACVPVQEEEEEVRLTLP 70
RAT R1     -----MSSHKGS----AVAQGNGAPSSNREADTVELAE LGPLLEEK GKRAATSPAKAEEEQACVPVQEEEEEVRLTLP 70
Monkey R1  -----MSSHKGS----VVAQGNGAPASNREADTVELAE LGPLLEEK GKRVIANPPKAEEEQTCVPVQEEEEEVRLTLP 70
Human R2  MNEPTENRLGCSRTPEDIRLRKGHQLDGT RRGDNDSHQGDLEP ILEASVLSSSHKK SSEEHEYSDEAPQEDGFGMSPL- 81
Mouse R2  MNEPAKHRLGCTRTPEDIRLRKGHQLDDT RGSNNDNYQGDL EPSLET PVCSSYYENSPEEP ECHDDNSQEDGFGMSPL- 81
RAT R2     MNEPTENRLGCSRTPEDIRLRKGHQLDDT RGGNNDNHGDL EPSLET PVCSSYYENSPEEP ECHDDNSQEDGFGMSPL- 81
Monkey R2  MNEPTENRLGCSRTPEDIRLRKGHQLDAT RRGDNDSHQGDLEP ILEASVLSSSHKK SSEEHEYSDEAPQEDGFGMSPL- 81
          : . . : . : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
Human R1  LQAHHAMEKMEEFVYKVWEGRWVIPYDVLPDWLKDNDYLLHGH RPPMPSPFACF SIFRIHTETG 136
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RAT R1     LQAHHAMEKMEEFVYKVWEGRWVIPYDVLPDWLKDNDYLLHGH RPPMPSPFACF SIFRIHTETG 136
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Mouse R2  LQAHHAMERMEEFVCKVWEGRWVIPHDVLPDWLKDNDFLHGH RPPMPSPFACF SIFRIHTETG 147
RAT R2     LQAHHAMERMEEFVCKVWEGRWVIPHDVLPDWLKDNDFLHGH RPPMPSPFACF SIFRIHTETG 147
Monkey R2  LQAHHAMEKMEEFVCKVWEGRWVIPHDVLPDWLKDNDFLHGH RPPMPSPFACF SIFRIHTETG 147
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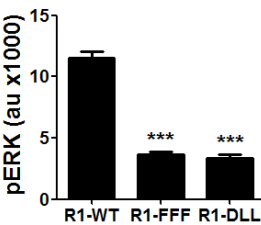
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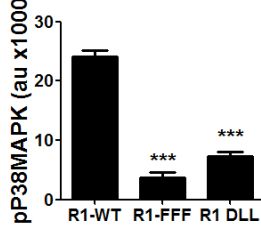
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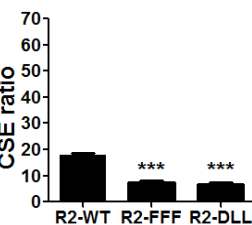
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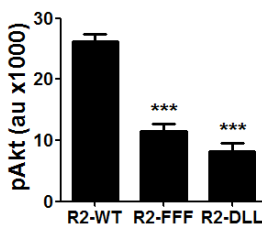
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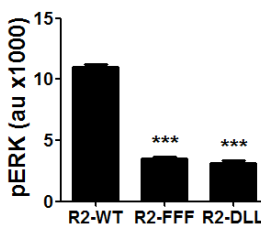
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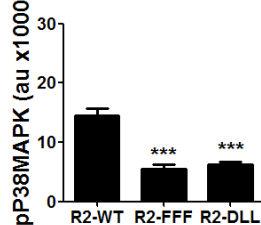
G



H



I



Highlights

- AdipoR1/R2 exhibit different cell-surface expression and temporal signalling profiles
- The non-conserved, N-terminal trunks (AdipoR1₍₁₋₇₀₎/R2₍₁₋₈₁₎) underpin the differences
- Conserved trafficking motifs are required for cell-surface expression of both receptors

Figure 1

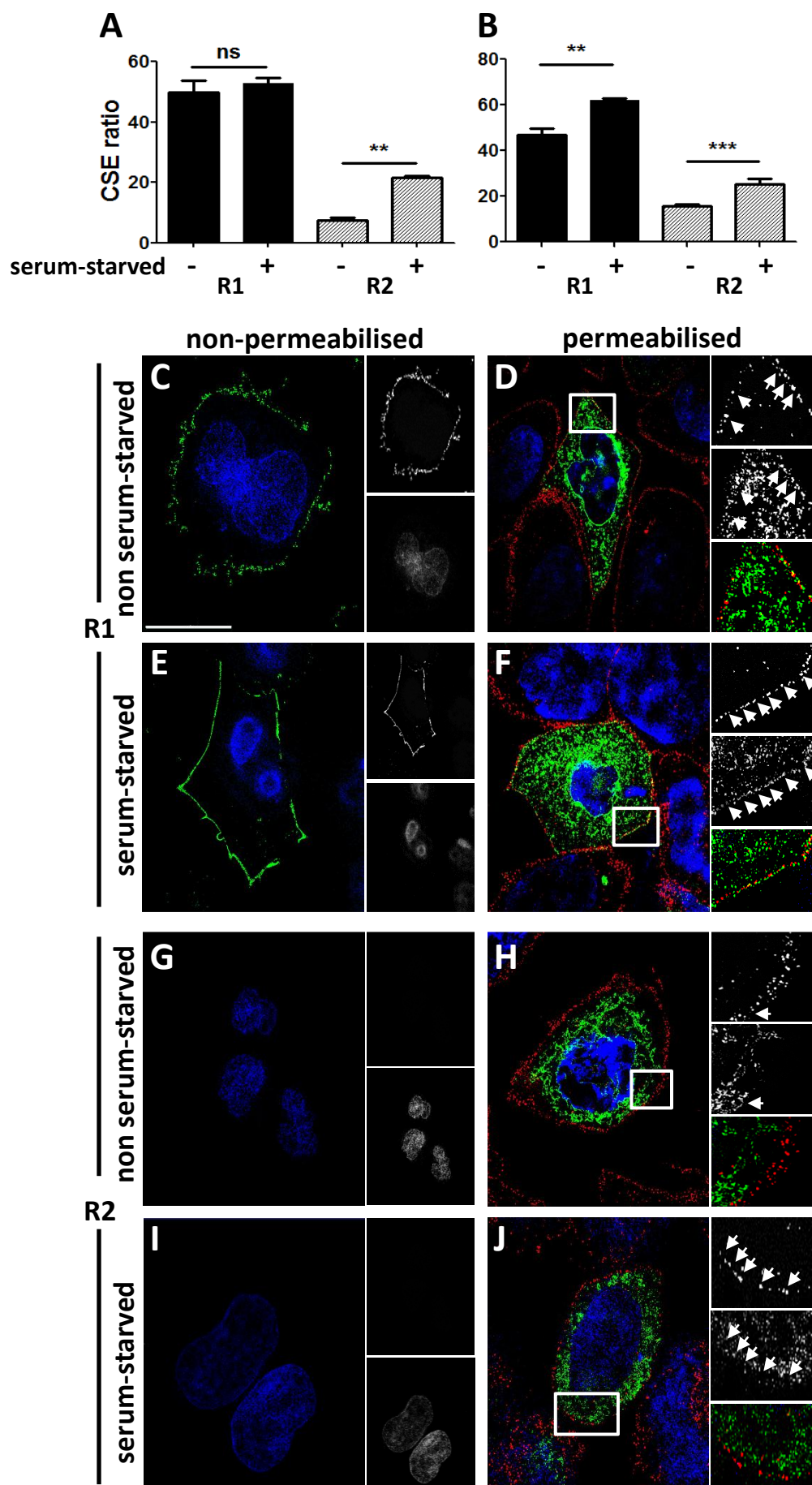


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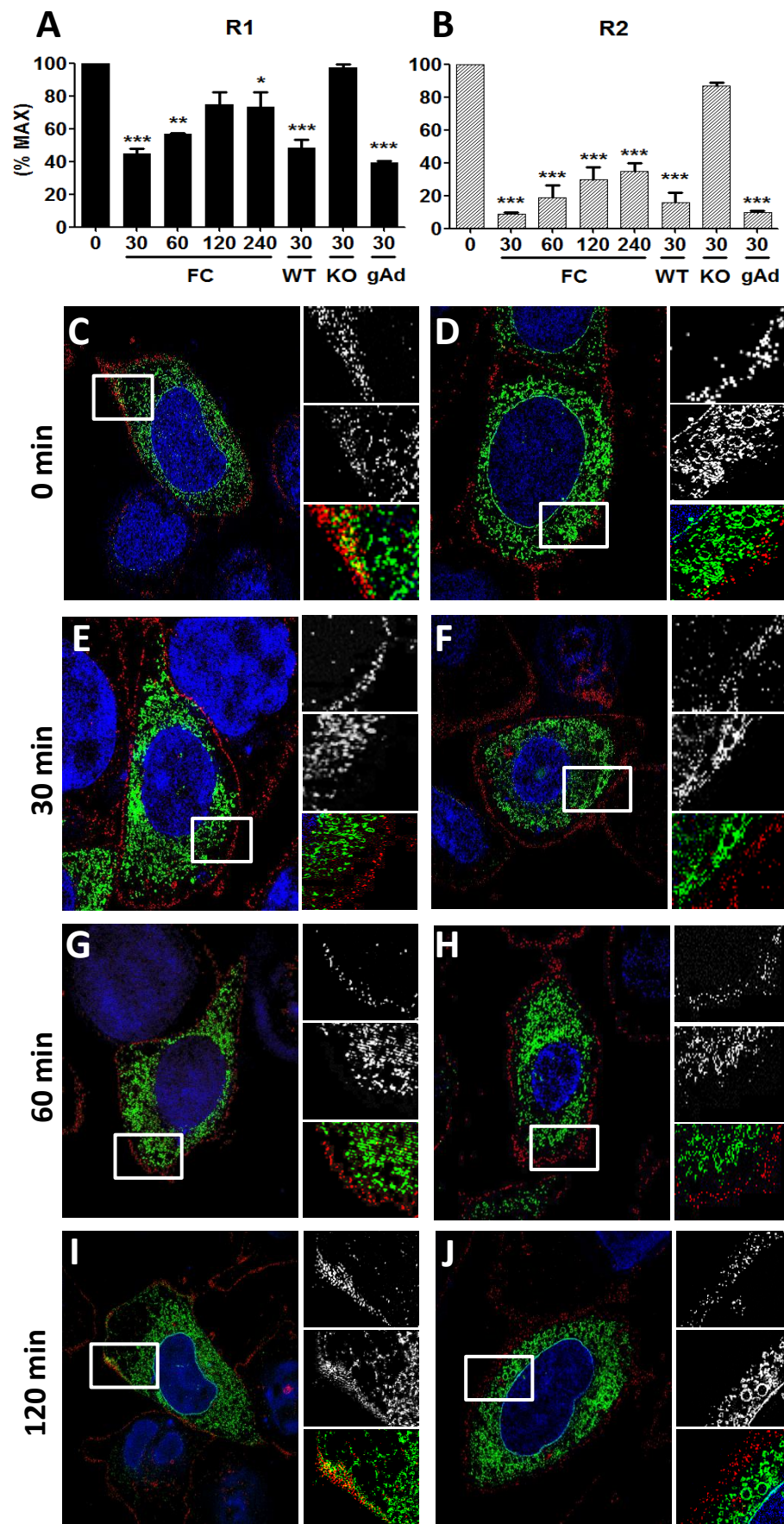


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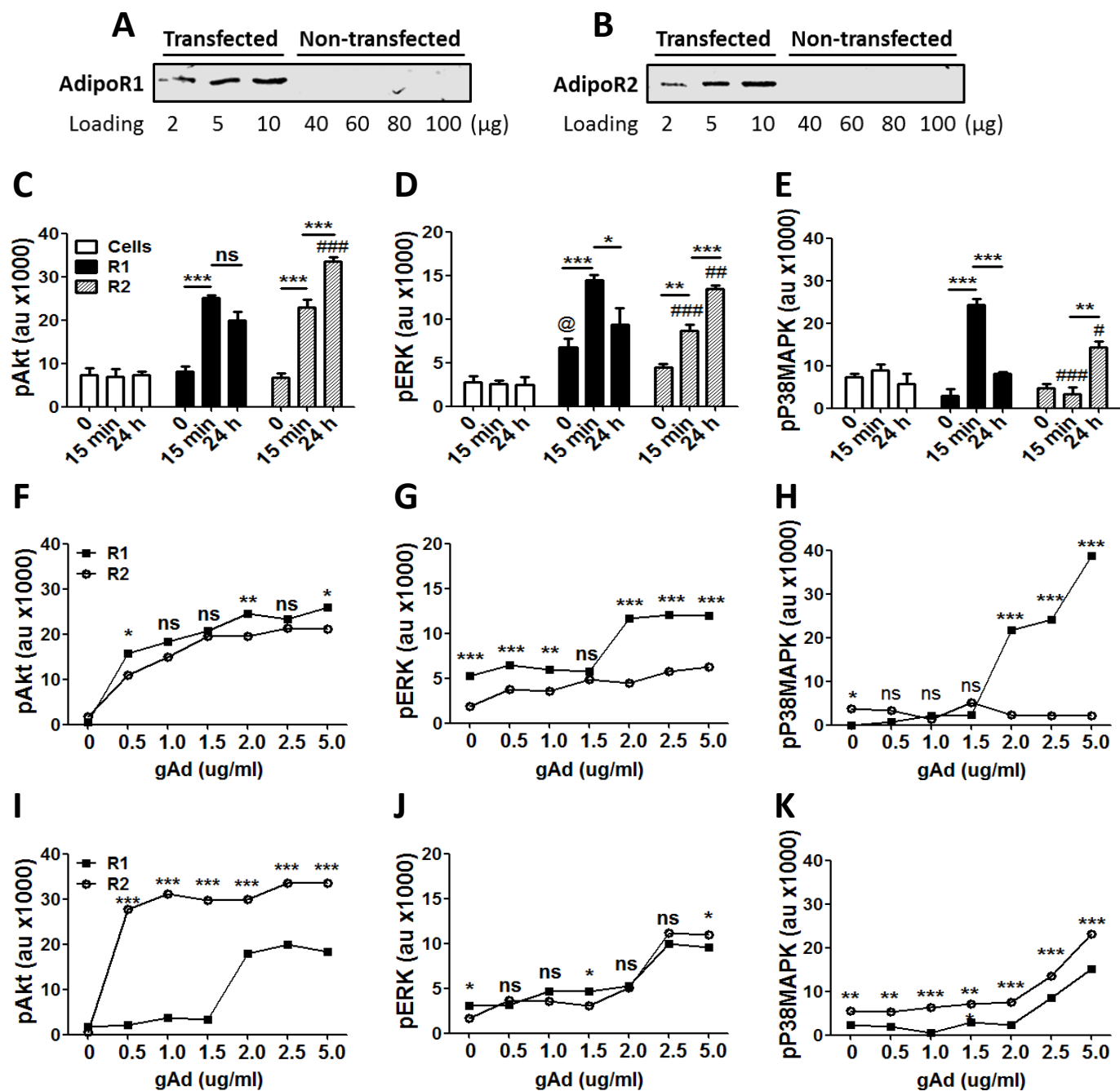


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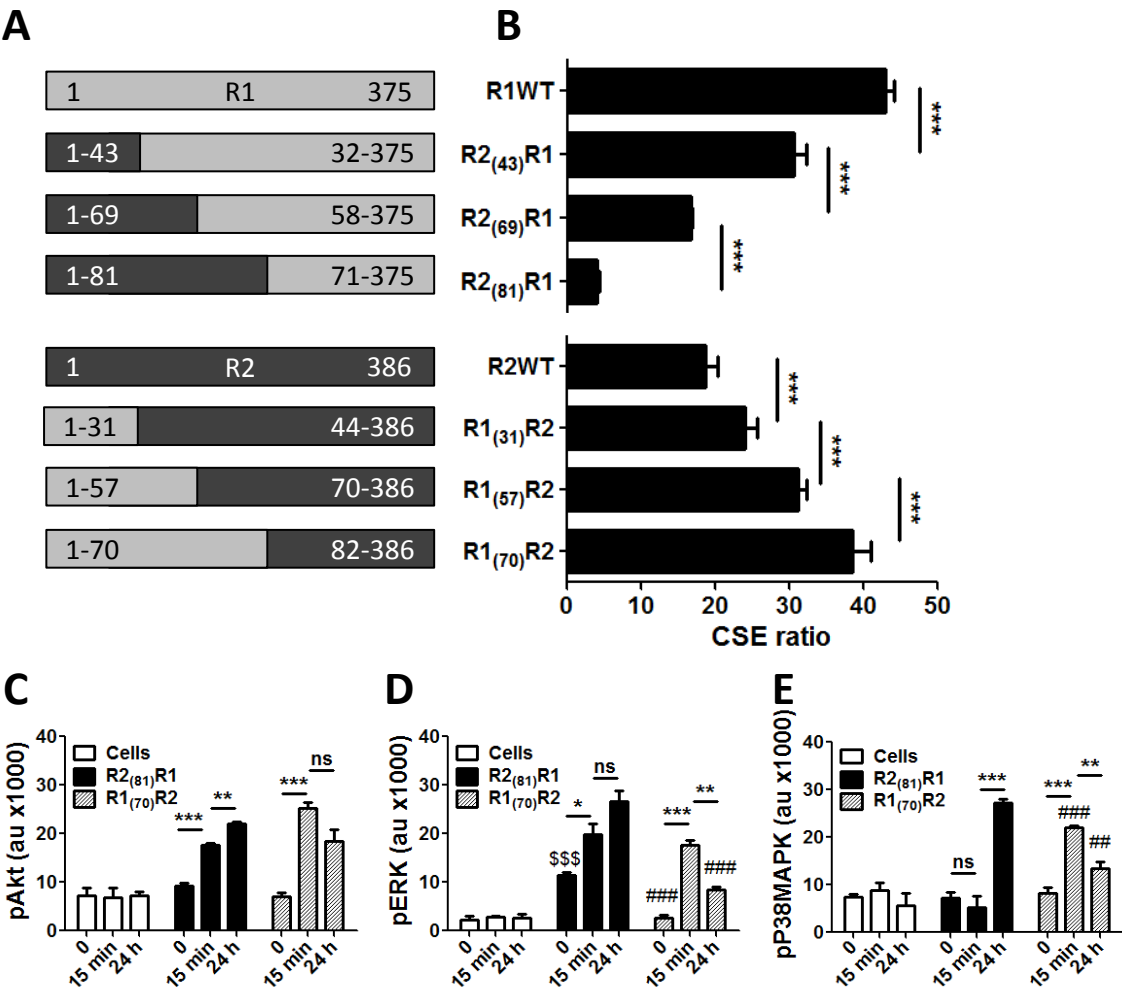
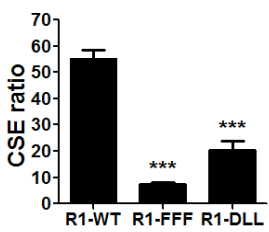


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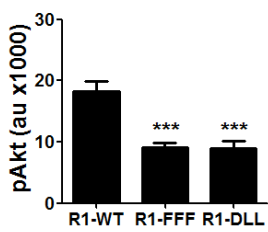
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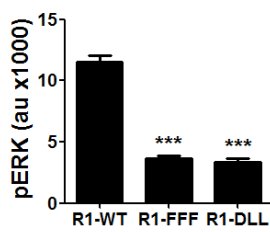
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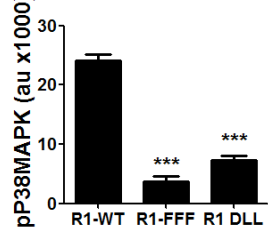
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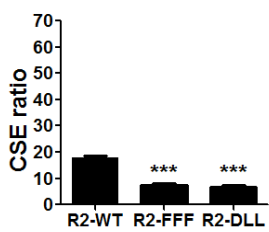
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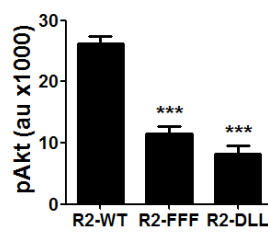
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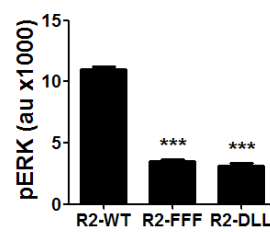
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G



H



I

